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Completion of the nucleotide sequence of the central region of Tn5 confirms the presence of three resistance genes

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ABSTRACT

The DNA sequence of the region located downstream from the kanamycin resistance gene of Tn5 up to the right inverted repeat IS50R has been determined. This completes the determination of the sequence of Tn5 which is 5818 bp long. The 2.7 Kb central region contains three resistance genes : the kanamycin-neomycin resistance gene, a gene coding for resistance to CL990 an antimitotic-antibiotic compound of the bleomycin family and a third gene that confers streptomycin resistance in some bacterial species but is cryptic in E. coli. A Tn5* mutant able to express streptomycin resistance in E. coli was isolated. With this mutant, it was demonstrated that in E. coli the expression of the three resistance genes is coordinated in a single operon.

INTRODUCTION

Transposon Tn5 (recently reviewed by Berg and Berg(1)) was originally isolated as a kanamycin-neomycin resistance determinant present in a Klebsiella resistance factor (2). It is composed of a central region of 2.7 kilobase pairs (Kb) flanked by two inverted repeats of 1.5 kb, the insertion sequences IS50, denoted respectively IS50L (left) and IS50R (right). IS50R codes for two proteins translated from a single reading frame. One of these is a transposase, the other an inhibitor of transposition (3,4). IS50L differs by one base pair from IS50R. This single change has two consequences: (i) it produces an ochre mutation that truncates both proteins which then, become inactive; (ii) it creates a good promoter P for the kanamycin resistance gene located in the adjacent region of the central part of Tn5 (5,6).

Only the kanamycin resistance function was known for Tn5 until 1981 when a streptomycin resistance unexpressed in E. coli was evidenced in Methylobacterium organophilum (7) and later confirmed in Rhizobium (8) and in other non-enteric bacteria (9). The streptomycin resistance gene was located in a 1000 bp segment of the central region, 300 bp downstream from the kanamycin resistance gene (10). The direction of transcription of

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kanamycin and streptomycin resistance genes was shown to be identical (8,10,11). In addition, two different aminoglycoside phosphotransferase activities were separated in crude extracts of M. organophilum (Tn5) (10). One corresponded to the aminoglycoside phosphotransferase (3') type II (APH(3')II) the only aminoglycoside modifying enzyme expressed by Tn5 in E. coli. The other was a streptomycin phosphotransferase (SPH). The two activities almost coeluted from a gel filtration column, the SPH being slightly retarded.

Recently G. Tiraby (Université Paul Sabatier, Toulouse, France) showed that Tn5 codes also for a resistance against a glycopeptide of the bleomycin family, called compound CL990 (to be patented). This new resistance gene, which is expressed in E. coli, was located in a 400 bp DNA segment immediately downstream from the kanamycin resistance gene (G. Tiraby, personal communication). Independently, Genilloud et al found that Tn5 encoded bleomycin resistance in the aforementioned region (12).

DNA sequence of the IS50s was achieved by Auerswald et al (13). In the central region, the kanamycin resistance gene and 360 nucleotides downstream from it were sequenced by Beck et al (14). The completion of the sequence of the central region presented in this paper provides information on the precise location of the resistance genes mentioned above. Moreover, their expression is demonstrated to be organized in a single operon with the aid of a Tn5* mutant able to express streptomycin resistance in E. coli.

MATERIALS AND METHODS

Source of DNA :

Recombinant plasmids pPM111, pPM116 and pPM122, previously constructed to localize the streptomycin resistance gene in Tn5 (10), were used as sources of DNA. They contained fragments of Tn5 cloned into plasmid pBR322. The initial source of Tn5 was plasmid pRZ102 (15). Plasmids were purified from E. coli HB101 (16) and E. coli FC1 (10).

Purification of plasmid DNA :

Plasmid DNA, extracted by the clear lysate method, was purified on cesium chloride gradients as described by Humphreys et al. (17).

Nucleotide sequence determination :

Fragments were 3'-end-labeled with {α-^{32}P} dXTP using DNA polymerase (large fragment) (18). Restriction fragments were separated on thin polyacrylamide gels at 6% or 8% and then eluted overnight by diffusion

(19). Strand separation was performed essentially as described in (19) except that 50% dimethylsulfoxide was used instead of 30% and that samples were eluted overnight.

End-labeled restriction fragments were subjected to base specific chemical cleavages according to Maxam and Gilbert (20). The G, A+G, A+C, C, C+T reactions were used, and the products were analysed on 20% and 8% or 6% polyacrylamide thin gels containing 8M urea (21). 6% polyacrylamide sequencing gels were dried in order to enhance band sharpness and to shorten exposure time (22).

Comparison of amino acid sequences :

The sequences of amino acids were compared at the Calcul Center of Institut Pasteur, Paris, using the algorithm of Sellers (23), as generalized by Waterman *et al.* (24) and Smith *et al.* (25). The choice of the deletion weight was three for each gap plus 1.5 times the number of residues in each gap; i.e., for a gap of length k the gap penalty Wk was 3 + 1.5 k. The range of distance values for a pair of sequence elements varies from 0 to 3.

Media and chemicals :

Bacteria were grown in LB medium (26). Restriction endonucleases were from Boehringer or Biolabs. They were used as suggested by the suppliers. DNA polymerase (large fragment) was from Biolabs. {α-^{32}P} deoxynucleotides (3000 Ci/mMole) were from Amersham. CL990, an antimitotic product of Streptomyces spp. was kindly given by G. Tiraby.

Genetic techniques :

Techniques of transformation and analysis of transformants were already described (10).

Selection of a Tn5 mutant able to express streptomycin resistance in E. coli :

Such a mutant was obtained with pPM111, a pBR322 derivative containing a fragment *Hin*dIII-*Bgl*II from Tn*5* (10). This fragment includes the whole central region of Tn*5* and the kanamycin promoter P (Fig. 1). A plasmid pPM109 of similar structure, but without the major part of the streptomycin gene (10), was used as a control. On LB medium with streptomycin (20 μg/ml), resistant colonies of *E. coli* FC1(pPM111) appeared at a frequency of 2.10^{-6}. This frequency was lower than 10^{-7} with *E. coli* FC1(pPM109). Plasmids were purified from several Sm^R clones. Restriction analysis of pPM111 and of pPM111* (i.e., Sm^R) were identical.

Streptomycin phosphotransferase, assayed as described previously

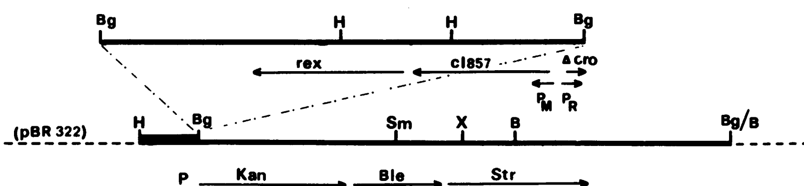

Figure 1 : Construction of plasmid pPM114. The lower part of the figure shows pPM111 previously constructed by insertion into pBR322 of the whole central region of Tn5 and of the promoter P (10). The upper part of the figure shows the BglII fragment of λ cI857 inserted into pPM111 to obtain pPM114. The fragment BglII of λ includes the genes rex, cI, deleted cro and the two promoters P_M and P_R. In pPM114 the kan gene is under the control of P_R which is regulated by the thermosensitive repressor coded by cI857.
Restriction sites are indicated by letters; H : HindIII; Bg : BglII; Sm : SmaI; X : XhoI ; B : BamHI.

(10), was detected in crude extracts of E. coli FC1(pPM111*) but not in crude extracts of E. coli FC1(pPM111). After transformation of E. coli FC1 with the DNA of pPM111*, streptomycin resistance was cotransferred with the plasmid markers Amp^R and Km^R.

Construction of plasmid pPM114 with the kanamycin resistance gene under the control of the promoter P_R of λ :

DNA of λ cI857 was digested by BglII. After electrophoresis, the 2400 bp fragment carrying the P_R promoter was electroeluted and ligated to pPM111 digested by BglII (Fig. 1). After transformation into E. coli FC1, clones which were kanamycin resistant at 37°C and kanamycin sensitive at 30°C were selected and purified. Restriction analysis of plasmid pPM114 conferring the latter phenotype, showed that the BglII λ-DNA fragment was inserted according to the orientation shown on Fig.1. The same construction was achieved with pPM111* (plasmid pPM114*).

RESULTS

DNA sequence of the central part of Tn5

The sequencing strategy is shown in Figure 2. The sequence was determined from the unique SmaI site of Tn5 up to the BglII site of IS50R. The first 180 bp and the last 80 bp overlap with the DNA sequences published earlier (13,14) and are in complete agreement with them. The sequence of Tn5, presented in Figure 3, starts at position 2301 (according to Beck's numbering system) (14) and extends up to position 4340 i.e. 25 bp downstream from the BglII site of IS50R. Tn5 is then 5818 bp long, a

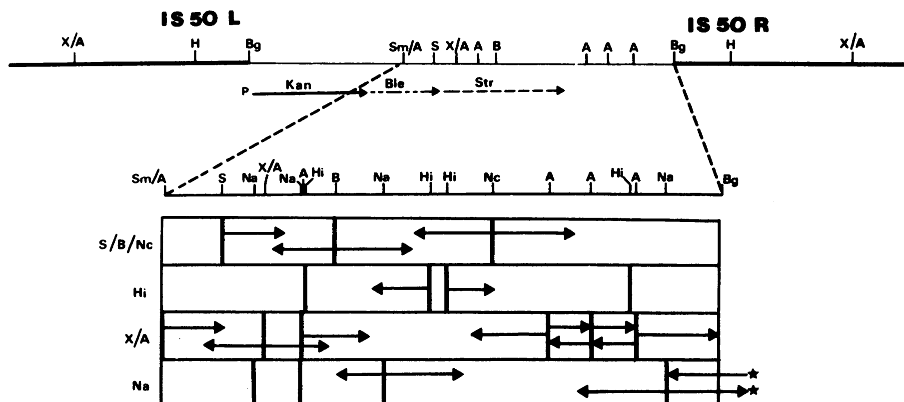

Figure 2 : Physical map of Tn5 and sequencing strategy.
Upper part : restriction map of Tn5 and localization of the resistance genes for kanamycin, bleomycin and streptomycin. P : promoter of the kanamycin resistance gene.
Lower part : DNA segment actually sequenced in this work and sequencing strategy. The arrows indicate the direction of sequencing and the length of the sequence determined.
A: AvaI, X: XhoI, H: HindIII, Bg: BglII, Sm: SmaI, S: SalI, B: BamHI, Na: NarI, Hi: HinfI, Nc: NcoI. The three latter sites are indicated only in the sequenced fragment. The * indicates that the NarI site is located in pBR322 which was used as subcloning vector.

value which is in good agreement with the estimated value of 5.7 kb (2).

Precise location of the three resistance genes of the central region of Tn5.

Analysis of the DNA sequence of the central region of Tn5 revealed the existence of two open reading frames (ORF) downstream from the kanamycin resistance gene. Actually, 20 bp after the stop codon of the latter gene, an ORF of 378 bp was found. It was preceded at a relatively long distance by a putative ribosome binding site GGA, 14 bp before the ATG and closed by a TGA opal codon (position 2744). This ORF is assumed to be the ble gene which codes for resistance to compound CL990. Indeed, this gene has been localized between the kanamycin resistance gene and the unique XhoI site of the central region (G. Tiraby, personal communication). These results were further confirmed in our laboratory (data not shown) with the aid of different recombinant plasmids carrying various fragments of Tn5 and with a set of IS21 insertions in Tn5 (10). A recent study of Genilloud et al. (12) including the identification of the ble gene product brought a final argument for the existence of the ble gene.

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                                                                MetThrAspGlnAlaThrProAsnLeuProSerArgAspPheAspSerThrAlaAla
TCGCAGCGCATCGCCTTCTATCGCCTTCTTGACGAGTTCTTCTGAGCGGACTCTGGGGTTCGAAATGACCGACCAAGCGACGCCCAACCTGCCATCACGAGATTTCGATTCCACCGCCG
                                                                                                    2400
 PheTyrGluArgLeuGlyPheGlyIleValPheArgAspAlaGlyTrpMetIleLeuGlnArgGlyAspLeuMetLeuGluPhePheAlaHisProGlyLeuAspProLeuAlaSerTrp
CCTTCTATGAAAGGTTGGCTTCGGAATCGTTTTCCGGGACGCCGGCTGGATGATCCTCCAGCGCGGGGATCTCATGCTGGAGTTCTTCGCCCACCCCGGGCTCGATCCCCTCGCGAGTT
                                                                                    2500          SmaI
 PheSerCysCysLeuArgLeuAspAspLeuAlaGluPheTyrArgGlnCysLysSerValGlyIleGlnGluThrSerSerGlyTyrProArgIleHisAlaProGluLeuGlnGluTrp
GGTTCAGCTGCTGCCTGAGGCTGGACGACCTCGCGGAGTTCTACCGGCAGTGCAAATCCGTCGGCATCCAGGAAACCAGCAGCGGCTATCCGCGCATCCATGCCCCCGAACTGCAGGAGT
                                                      2600                                                   PstI
 GlyGlyThrMetAlaAlaLeuValAspProAspGlyThrLeuLeuArgLeuIleGlnAsnGluLeuLeuAlaGlyIleSer
GGGGAGGCACGATGGCCGCTTTGGTCGACCCGGACGGACGCTCCTGCGCCTGATACAGAACGAATTGCTTGCAGGCATCTCATGAGTGTGTCTTCCCGTTTTCCGCCTGAGGTCACTGC
                   SalI      2700
   MetGluArgTrpArgLeuLeuArgAspGlyGluLeuLeuThrThrHisSerSerTrpIleLeuProValArgGlnGlyAspMetProAlaMetLeuLysValAlaArgIleProAsp
GTGGATGGAGCGCTGGCGCCTGCTGCGCGACGGCGAGCTGCTCACCACCCACTCGAGCTGGATACTTCCCGTCCGCCAGGGGGACATGCCGGCGATGCTGAAGGTCGCGCGCATTCCCGA
              2800                                AvaI                                                                2900
 GluGluAlaGlyTyrArgLeuLeuThrTrpTrpAspGlyGlnGlyAlaAlaArgValPheAlaSerAlaAlaGlyAlaLeuLeuMetGluArgAlaSerGlyAlaGlyAspLeuAlaGln
TGAAGAGGCCGGTTACCGCCTGTTGACCTGGTGGGACGGCAGGGCGCCGCCCGAGTCTTCGCCTCGGCGGCGGGCGCTCTGCTCATGGAGCGCGCGTCCGGGGCCGGGGACCTTGCACA
                                                 AvaI                                         3000
 IleAlaTrpSerGlyGlnAspAspGluAlaCysArgIleLeuCysAspThrAlaAlaArgLeuHisAlaProArgSerGlyProProProAspLeuHisProLeuGlnGluTrpPheGln
GATAGCGTGGTCCGGCCAGGACGACGAGGCTTGCAGGATCCTCTGCGACACCGCCGCTCGTCTGCACGCGCCGCGGTCCGGACCGCCGCCCGATCTCCATCCGCTACAGGAATGGTTCCA
                                      BamHI                                         3100
 ProLeuPheArgLeuAlaAlaGluHisAlaAlaLeuAlaProAlaAlaSerValAlaArgGlnLeuLeuAlaAlaProArgGluValCysProLeuHisGlyAspLeuHisHisGluAsn
GCCGCTTTTCCGGTTGGCCGCTGAGCACGCGGCACTTGCGCCCGCCGCCAGCGTAGCGCGCCAACTTCTGGCGGCGCCGCGCGAGGTGTGCCCGCTCCACGGCGACCTGCACCACGAGAA
                                                      3200
 ValLeuAspPheGlyAspArgGlyTrpLeuAlaIleAspProHisGlyLeuLeuGlyGluArgThrPheAspTyrAlaAsnIlePheThrAsnProAspLeuSerAspProGlyArgPro
CGTGCTCGACTTCGGCGACCGCGGCTGGCTGGCCATCGACCCGCACGGACTGCTCGGCGAGCGCACCTTCGACTATGCCAACATCTTCACGAATCCCGATCTCAGCGACCCCGGTCGCCC
                                3300
 LeuAlaIleLeuProGlyArgLeuGluAlaArgLeuSerIleValValAlaThrThrGlyPheGluProGluArgLeuLeuArgTrpIleIleAlaTrpThrGlyLeuSerAlaAlaTrp
GCTTGCGATCCTGCCGGGCAGGCTGGAGGCTCGACTCAGCATTGTGGTCGCGACGACCGGGTTTGAGCCCGAACGGCTTCTTCGCTGGATCATTGCATGGACGGGCTTGTCGGCAGCCTG
              3400                                                                                                    3500
 PheIleGlyAspGlyAspGlyGluGlyGluGlyAlaAlaIleAspLeuAlaValAsnAlaMetAlaArgArgLeuLeuAsp
GTTCATCGGCGACGGCGACGGCGAGGGCGAGGGCGCTGCGATTGATCTGGCCGTAAACGCCATGGCACGCCGGTTGCTTGACTAGCGCGGTCACCGATCTCACCTGGTCGTCGAGCTAGG
                                                                                                    3600
TCAGGCCGTGTCGGGCGTGATCCGCTGGAAGTCGTTGCGGGCCACACCCGCCGCCTCGAAGCCCTGCACCAGGCCGGCATCGTGGTGTGCGTGGCCGAGGGACTATGGAAGGTGCCGGAC
                                                                                    3700
GATCTGCCCGAGCAGGGCCGCCGCTATGACGCCCAGCGTCTTGGTGGCGTGACGGTGGAGCTGAAATCGCACCTGCCCATCGAGCGGCAGGCCCGCGTGATCGGTGCCACCTGGCTTGAC
      AvaI                                                    3800
CAGCAGTTGATCGACGGTGGCTCGGGCTTGGGCGACCTGGGCTTTAGCAGTGAGGCCAAGTAGGCGATACAGCAGCGCGCGGACTTCCTGGCCGAACAGGGACTGGCCGAGCGGCGCGGG
                   AvaI         3900
CAGCGCGTGATCCTCACCGGAATCTGCTCGGCAGCAGCGGGCTCGGGAACTGGCGCAGGCCGCGAAGGACATTGCCGCCGATACCGGCCTGGAGCATCGCCCCGTGGCCGACGGCCAGCG
              4000                        AvaI                                                                        4100
CGTTGCCGGCGTCTACCGGCGCCCCGTCATGCTCGCCAGCGGGCGAAATGGGATGCTTGATGACGCCAAGGGGTCCAGCCTCGTGCGGTGGAAGCCCATCGAACAGCGGCTTGGGGAGCCA
                                                                                                    4200
GCTCGCCGCGACGGTGCGCGGTGGCGGCGTGTCTTGGGAGATTGGACGACAGCGTGGGCCGGCCCCTGTCTCTTGATCAGATCTTGATCCCCTGCGCCATCAGATCCTTGGCGGCAAGAA
                                                                                    4300
                                                                                    BglII
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Downstream from the ble gene, 38 bp farther, at position 2785, a long ORF of 798 bp could be detected. It is preceded by a Shine and Dalgarno sequence GAGG, 11 bp before the initiation codon. This ORF is assumed to be the streptomycin resistance gene. However, this gene might also start 42 bp upstream at position 2743. In that case, the ATG of the str gene would overlap with the TGA stop codon of the ble gene. Furthermore, a polypeptide starting at the first ATG (position 2743) would have a molecular weight of 30,607 slightly higher than the molecular weight of 29,053 deduced for the kan gene product (14). Translation starting at the second ATG (position 2785) would yield a polypeptide with a molecular weight of 29,033 more in accordance with previous gel filtration data which showed that the SPH had an apparent molecular weight slightly lower than the APH(3')II.

Whatever the exact starting point of the streptomycin resistance gene, the determination of the total sequence of Tn5 ruled out the ATG in position 2672 as a possible start for the str gene as considered once (8). This ATG codes for a methionine located in the sequence of the ble gene and it is followed 69 bp downstream by the TGA stop codon of this gene. This result agrees with previous reports (11,27) which showed that a deletion of the region located on the left side of the SalI site does not prevent the expression of the streptomycin resistance in *Rhizobium meliloti*.

In summary, the complete sequence of the central region of Tn5 showed that the three resistance genes for kanamycin, bleomycin and streptomycin were encoded respectively by three different ORFs.

Coordinated expression of the three resistance genes of Tn5

The question as to whether the expression of the three resistance genes is coordinated in a single operon received a preliminary answer with the aid of a Tn5* mutant able to express streptomycin resistance in *E. coli*. Results of the expression of the three resistance genes are reported in Table 1. In pPM111 and pPM111*, the kanamycin gene is under the control of its natural promoter P and gene expression was not altered by growth temperature 30°C or 37°C. In pPM114 and pPM114* the central region of Tn5

Figure 3 : Nucleotide sequence of the central region of Tn5. The numbers indicate the position of the nucleotides starting from the IS50L 5' end. Sequences bracketed were published (12,13). The deduced aminoacid sequence for the proteins encoded is indicated. The putative ribosome binding sites are underlined with a broad line. The nonsense codons at the end of each of the three resistance genes are boxed.

Table 1 :
THERMO-REGULATION OF THE THREE RESISTANCE GENES OF TN5 IN pPM114*

	Minimal Inhibitory Concentration (µg/ml) with :					
	Kanamycin		CL990		Streptomycin	
Growth temperatures :	30°	37°	30°	37°	30°	37°
E. coli FC1 with :						
pPM 111	>500	>500	>500	>500	2	2
pPM 111*	>500	>500	>500	>500	20	20
pPM 114	10	>500	2	>500	2	2
pPM 114*	10	>500	2	>500	4	30

was placed under the control of the P_R promoter of λ regulated by the thermosensitive repressor cI857. In that case, the expression of the three resistance genes in E. coli became simultaneously temperature inducible. This result suggests that the three resistance genes are under the control of a single promoter.

Gene products of the central region of Tn5

Comparisons of the amino acid sequences of the aminoglycoside phosphotransferases responsible for kanamycin resistance and encoded by Tn5, Tn903 and S. fradiae have indicated a statistically certifiable relationship between these enzymes of distant sources (14,28,29). The same comparative statistical analysis, performed between the sequences of those enzymes and the protein encoded by the str gene did not reveal any homology.

The ble gene codes for a polypeptide of 14,058 daltons which has been observed by polyacrylamide gel electrophoresis after synthesis in "maxi-cells" (12). Comparison of its amino acid sequence, deduced from the DNA sequence, with protein sequences of the National Biomedical Research Foundation data base (N.B.R.F., release I), did not give any clue to the nature of this polypeptide. The mode of resistance to CL990 is still unknown. No adenylation, acetylation or phosphorylation of compound CL990 was detected in crude extracts of E. coli::Tn5 by phosphocellulose paper binding assay (30).

DISCUSSION

Tn5 is a transposon of 5818 base pairs composed of two IS50s and of a central region. Completion of DNA sequence determination and other studies

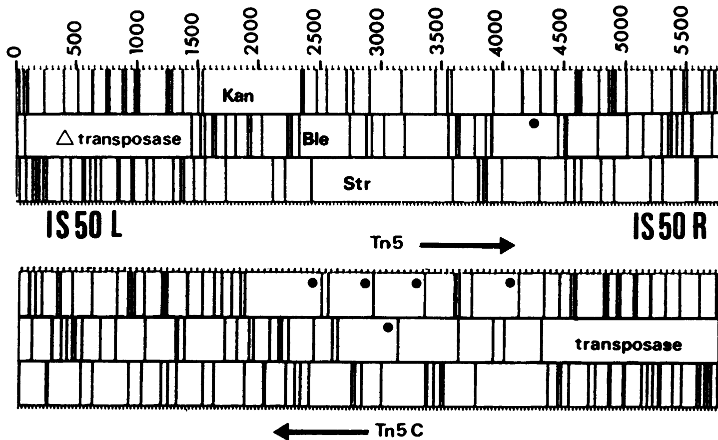

Figure 4 : Schematic drawing of the 3 reading frames on both strands of Tn5. Genes for transposase active or truncated (Δ) (3,4) and resistance genes to kanamycin, CL990 and streptomycin are located in their respective ORF. ORFs longer than 300 bp, starting with an ATG are marked with a dot.

(10, 12, 14, 15) showed that the central region comprises three genes coding for three polypeptides of 29,053, 14,038 and 29,033 daltons respectively. As no rho independent or dependent termination signals (31) or palindromic unit (32) could be detected downstream from the streptomycin resistance gene, we looked for other open reading frames on the same strand and on the other strand. On the same strand, the longest ORF starting with an ATG is 300 bp long (Fig.4). On the complementary strand, one can detect five ORFs of reasonable size (between 300 and 500 bp); but four are located in a region where the other strand codes for the resistance genes. No data are available concerning these ORFs and no further attempt was made to assign any function to these putative genes.

The codon usage of the three identified genes is identical to that of *E. coli* structural genes, i.e. it did not show any preference for codons corresponding to abundant tRNAs as in ribosomal proteins (33). Nor did it show usage of rare codons. The only peculiarity was a preference for G or C on the third position of the codon compared to A or T, a fact to be correlated with the relatively high GC content of Tn5 : 60% with individual values of 55% for the IS50s and 65% for the central region.

The building process of the operon of the central region of Tn5 during the course of evolution presents a particular interest as it gathers genes without apparent physiological links. Emergence of Tn5 might

have occured either by successive acquisition of the kan, ble and str genes, either in one time by mobilization of the whole operon region by two IS50 elements. The latter evolutionary pattern would be in accordance with the hypothesis that aminoglycoside resistance genes derived from antibiotic producing organisms and with the fact that these organisms usually carry several resistance genes (34). The respective GC contents of kan (60%), ble (62%), str (69%) and of the remaining part of the central region (68%) suggests rather a two-step evolution (i) by mobilization of kan and ble by two IS50s (ii) by acquisition of str. In that case, the hypothetical Tn5 ancestor without str, remains to be found.

The sequence of the streptomycin resistance gene does not give a straightforward explanation for its non expression in the Enterobacteriaceae family. The sequences of Tn5 wild type and Tn5* mutants able to express streptomycin resistance in E. coli should provide information on that question.

Knowledge of the sequence of the ble gene and its vicinity should allow easier subcloning of this resistance in expression vectors. Indeed CL990 and bleomycin are powerful antimitotic compounds and expression of the ble gene might provide a good positive selection marker in eukaryotes.

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